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Effects of Marine Bacteria on the Culture of Axenic Oyster *Crassostrea gigas* (Thunberg) Larvae

PHILIPPE DOUILLET[1] AND CHRISTOPHER J. LANGDON

Oregon State University, Department of Fisheries, Hatfield Marine Science Center, Newport, Oregon 97365

Abstract. Bacteria-free oyster larvae (*Crassostrea gigas*) were cultured under aseptic conditions; they were fed axenic algae (*Isochrysis galbana*), and the medium was inoculated with isolated strains of marine bacteria. Twenty-one bacterial strains were tested, and most were detrimental to larval survival and growth. However, additions of strain CA2 consistently enhanced larval survival (21–22%) and growth (16–21%) in comparison with control cultures that were fed only algae. Size-frequency distributions of populations of larvae cultured for 10 days on axenic algae were skewed due to the poor growth of many individuals; whereas size-frequencies from populations of larvae fed axenic algae supplemented with CA2 bacteria were distributed normally. Strain CA2 may therefore make a nutritional contribution to the growth of oyster larvae. *I. galbana* did not grow under the light intensities used for larval culture; thus the improvement in larval growth cannot be attributed to bacterial enhancement of algal growth and, consequently, food availability. Naturally occurring microflora from Yaquina Bay, Oregon, depressed survival or growth of larvae-fed live algae.

Introduction

Bivalve larvae in culture vary substantially in survival and growth (Davis, 1953; Loosanoff, 1954; Walne, 1956a). Between 25% and 50% of the variability in the growth of a single population of mussel larvae (Innes and Haley, 1977), or different populations of larval *Crassostrea virginica* (Newkirk *et al.*, 1977), are due to genetic factors. A significant proportion of the variability in the survival of *C. gigas* larvae was similarly attributed to genetic factors (Lannan, 1980). Exogenous factors, such as temperature (Loosanoff, 1959), salinity (Bayne, 1965), pH (Calabrese and Davis, 1970), food quantity (Walne, 1965), food quality (Davis, 1953), age of the algal food (Dupuy, 1975), larval concentration (Loosanoff *et al.*, 1953), size of container (Dupuy, 1975), silt (Davis and Hidu, 1969), exudates of unfavorable algal species (Bayne, 1965), water quality (Millar and Scott, 1967) and toxicants (Walne, 1970) have been found to contribute significantly to variability in larval growth. Nonetheless, even different cultures of larvae obtained from the same parents and grown under identical conditions of temperature, salinity and ration have been commonly reported to vary in their growth (Bayne, 1983).

The role of bacteria as beneficial or harmful agents in the culture of bivalve larvae has been the subject of many investigations, but this role has not been fully evaluated. Thirteen different isolates of marine bacteria did not support the growth of oyster larvae when provided as the sole source of particulate food (Davis, 1950, 1953). High bacterial densities in cultures of bivalve larvae are generally considered to be deleterious to the larvae (Walne, 1956a, 1956b, 1958), and even innocuous bacteria in large numbers have been reported to depress the rate of algal ingestion (Ukeles and Sweeney, 1969). Some bacterial strains are reportedly able to invade larvae, to produce toxins, or both (Guillard, 1959; Tubiash *et al.*, 1965; Tubiash *et al.*, 1970; Brown, 1973; Di Salvo, 1978; Nottage and Birkbeck, 1986). In contrast, bacteria have also been implicated as a food source for bivalve larvae (Carriker, 1956; Hidu and Tubiash, 1963) or as improving the growth of larvae fed on algae (Martin and Mengus, 1977; Beese, in Prieur *et al.*, 1990).

The elimination of microbial contaminants is prerequisite to a study of the effects of a bacterial strain on an organism in culture. This approach has been used to study the effects of several bacterial strains on cultures of the

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[1] Present address: The University of Texas at Austin, Marine Science Institute, P.O. Box 1267, Port Aransas, Texas 78373.

protozoan *Amoeba nitrophila* (Frosch, 1897 in Luck *et al.*, 1931); the cladoceran *Moina macrocopa* (Stuart *et al.*, 1931); and larvae of the clam *Mercenaria mercenaria* (Guillard, 1959).

In the present study, axenic larval *Crassostrea gigas*, obtained without the use of antibiotics, were used in a series of experiments meant to reveal whether selected strains of marine bacteria can consistently improve the survival and growth of algal-fed oyster larvae.

Materials and Methods

Maintenance of larvae, bacteria and algae

Bacteria-free oyster larvae were obtained according to the method of Langdon (1983). Adult oysters *Crassostrea gigas* were held at 18°C in a recirculating seawater system for a period of 4 to 6 weeks, depending on the initial reproductive condition of the broodstock. After this conditioning period, the oysters were opened and shucked. Using aseptic techniques in a laminar-flow hood, we disinfected the external surface of the gonads of each oyster with a 1% solution of sodium hypochlorite. A small incision was made through the surface of the gonads with a heat-sterilized scalpel, and gametes from each oyster were removed with sterile Pasteur pipettes and transferred to separate sterile flasks containing 0.2 μm-filtered, autoclaved seawater (FSSW). Eggs were fertilized by the addition of a few drops of sperm suspension and then were transferred to Erlenmeyer flasks containing FSSW at a density of 100 eggs ml^{-1}. Eggs were incubated on an orbital shaker at 25°C for 48 h. When the trocophore larvae had developed into veligers (straight-hinged larvae), subsamples of larvae were aseptically withdrawn for axenicity tests, and the remaining larvae were then held at 5°C for 5 days. Axenicity of larvae was determined by epifluorescence microscopy using 4′6-diamidino-2-phenylindole (DAPI) staining techniques (Porter and Feig, 1980). Samples of larvae were also added to 1/10 recommended concentration of Difco marine broth 2216 (3.74 g l^{-1}, salinity 30 ppt) and incubated at 25°C under aerobic or anaerobic conditions (BBL GasPak Pouch). Larvae from cultures that showed no evidence of microbial contamination from either the epifluorescence test or the 5 day broth incubations were considered adequate for experimentation. To confirm that the larvae were axenic, broth incubations were continued for 30 days. Axenic straight-hinged larvae were transferred to 250 ml Erlenmeyer flasks, each containing 150 ml of FSSW, closed with cotton plugs and capped with aluminum foil. Final larval density was 5 ml^{-1}. Growth experiments were then initiated by the addition to the culture flasks of axenic algae and the different bacterial strains. Shell lengths of 100 randomly selected larvae were measured, either with an optical micrometer fitted to a compound microscope, or with an image analysis system (Zeiss Videoplan 2).

Strains of marine bacteria were isolated from cultures of algae or oyster larvae at the Whiskey Creek Hatchery in Netarts Bay, Oregon. Other bacteria were isolated, either from the guts of adult oysters, or from incubations of protein capsules (Langdon, 1989) suspended in unfiltered seawater. Pure bacterial strains were obtained by the dilution method of Rodina (1972). Strains were grown, at 25°C, on marine agar 2216 or brain heart infusion agar (Difco). Bacteria grown on such solid media for 3 to 5 days were resuspended for 24 h in FSSW; they were then washed by centrifugation at 20,000 $\times$ g for 10 min and resuspended in FSSW.

Strains were added to larval cultures at concentrations of 10^5–10^6 cells ml^{-1}. Cell concentrations were derived from equations relating spectrophotometric absorbance (600 nm) and bacterial concentration; the latter value was determined by direct count after staining with DAPI (Porter and Feig, 1980). Such equations were developed and used for each strain tested.

Axenic *Isochrysis galbana* Parke (clone ISO) was obtained from the Culture Collection of Marine Phytoplankton (Maine). Algal cultures were grown at 20°C in 200 ml f/2 medium (Guillard and Ryther, 1962) illuminated by 1000–1500 lux of cool white fluorescent light under a 12 h light/12 h dark photoperiod. Algal axenicity was determined as described above for larvae.

All glassware was washed in 10% nitric acid, rinsed seven times with distilled water, and baked overnight at 450°C. Disodium ethylenediamine-tetraacetate (EDTA) was added at a final concentration of 1 ppm to all seawater to reduce the load of dissolved organic matter (Utting and Helm, 1985). Salinity of seawater after sterilization varied between 28 and 31 ppt. Heat sterilization was carried out for 15 min at 121°C and 1.06 kg cm^2 pressure.

Larvae fed on live algae and bacteria

Twenty-one marine bacterial isolates were tested in three culture experiments for their effects on the survival and growth of larvae fed axenic *Isochrysis galbana*. Experiment I included seven microbial isolates from the Whiskey Creek Hatchery (H1–H7) and five isolates from the guts of adult oysters (G1–G5). Control treatments were either larvae fed only algae or starved larvae.

In Experiment II, two strains (H6, H7) that improved larval growth in Experiment I were tested along with five strains isolated from the Whiskey Creek Hatchery (H8–H12), one strain isolated from the gut of an adult oyster (G6), and three strains isolated from protein capsules incubated in seawater (CA1–CA3). Control treatments included starved larvae and larvae fed only algae. In third control (SW), cultures of larvae were inoculated at the beginning of the experiment with naturally occurring bacteria present in 5 ml samples of 1 μm-filtered seawater collected from Yaquina Bay, Oregon. The larvae in the

third control treatment were fed axenic algae every other day. Experiments I and II were carried out with four replicates per treatment.

Experiment III was designed to retest strains that had enhanced larval survival and growth in Experiment II (H7, CA2). Control treatments similar to those described for Experiment II were included. Experiment III was carried out with eight replicates per treatment.

Cultures of bacteria-free oyster larvae (75.5–82 μm shell length) were inoculated once at the beginning of each experiment with bacterial strains. Bacteria-free algal cells, harvested from cultures in exponential growth phase, were added to the larval cultures every two days. The seawater of the larval cultures was not renewed during the culture period. The concentration of algal cells in each larval culture flask was estimated, as follows, before each feeding. A 2-ml sample of the larval culture medium was aseptically removed from each flask with a pipet; to prevent removal of larvae, the end of the pipet was covered with a 64 μm Nitex screen. Algal cells were preserved with formalin, concentrated by centrifugation, and re-suspended in 100 μl of 0.2 μm-filtered seawater. Algal concentrations in the samples were then determined with a hemocytometer. Fresh algae were then added to larval culture flasks to provide cell concentrations at pre-determined levels. Algal cell concentrations were increased by 15,000 cells ml^{-1}, from 40,000 to 100,000 cells ml^{-1} over a 10 day culture period. To provide uniform food quality during the experiments, algae from a single culture were added at each feeding period, to all larval cultures receiving an algal diet.

Larval culture flasks were placed randomly on orbital shakers in a temperature-controlled room at 25°C. The cultures were exposed to a light intensity of 50–70 lux for 12 h each day. No algal growth occurred at this low light intensity. After 10 days of culture, samples of water were aseptically withdrawn from flasks containing starved larvae or larvae fed only axenic algae; these samples were analyzed for microbial contamination as described above. The experimental data were analyzed only if these control treatments were bacteria-free at the end of the 10 day culture period.

Effects of CA2 bacteria on the growth of algae in larval cultures

Cells of axenic *I. galbana* were initially suspended at a concentration of 40,000 ml^{-1} in f/2 medium and then subdivided in sixteen 250 ml Erlenmeyer flasks. CA2 cells were added at 10^5 cells ml^{-1} (final concentration) to eight flasks, while FSSW was added to the other eight flasks to maintain similar initial algal concentrations in all flasks. The final volume of each algal culture was 200 ml. Four algal cultures inoculated with bacteria and four cultures that had received only FSSW were placed in conditions conducive to the growth of *I. galbana* (1000–1500 lux and 20°C); the remaining algal cultures were exposed to the conditions used for larval culture (50–70 lux and 25°C). The algal cultures were incubated on orbital shakers for three weeks. Every second day, 10 ml samples were removed aseptically from each algal culture, and algal concentrations determined with a Coulter counter (Model ZB1).

Larvae fed on dead algae and bacteria

Interactions between strain CA2 and living *Isochrysis galbana* that could modify algal food quality were not addressed in the previous experiments. To determine whether bacteria could enhance cultures of larvae fed on non-living diets, live *I. galbana* were replaced with dead algae.

In Experiment IV, known concentrations of axenic *I. galbana* were frozen at −5°C. Freezing and thawing broke the cell walls and membranes of the algal cells. Larvae were fed dead freeze-killed algae (FA) every two days according to the same protocol used with live algae. One group of larval cultures fed FA was maintained bacteria-free, and two groups were inoculated at the beginning of the experiment with either strain H6 at 10^5 cells ml^{-1} (final concentration), or with an inoculum of naturally occurring bacteria (SW). The wild strains were added in 5 ml samples of 1 μm-filtered seawater collected from Yaquina Bay, Oregon, at a concentration of 10^5–10^6 cells ml^{-1}. Other larval cultures received on alternate days, either additions of strain H6 (at a final concentration of 10^5 cells ml^{-1}) alone, or naturally occurring bacteria (SW) (5 ml of 1 μm-filtered seawater) alone. Control treatments included starved larvae and larvae fed every second day on live axenic *I. galbana*. Culture conditions and sample treatments were similar to those of experiments carried out with live algae. Four replicates were tested per treatment.

Algal cells were also killed by ^{60}Co-irradiation (5 megarads) at the Radiation Center at Oregon State University. Non-viability of irradiated algae (IA) was evident by the lack of growth of cells in f/2 medium at 20°C under 1000–1500 lux of fluorescent light emitted 12 h a day. The irradiation process also destroyed contaminants, as demonstrated by incubations, at 25°C, of irradiated algae in 1/10 diluted marine broth 2216 (3.74 g l^{-1}, salinity of 30 ppt) under either aerobic or anaerobic conditions (BBL GasPak Pouch). The integrity of the irradiated algal cells was verified by microscopic examination. Cell volumes of irradiated and non-irradiated algae from seven different cultures were determined with a Coulter counter (Model ZB1) equipped with a calibrated Coulter channelyser (Model 256).

To ensure that IA were acceptable to larvae as a food source, the ingestion rates of larvae fed on either IA or

live *Isochrysis galbana* were compared. Ingestion rates were calculated according to the methods described by Checkley (1980). Larval ingestion rates for live and ^{60}Co-irradiated algae were compared with a 2 sample t-test, after verifying homocedasticity by Cochran's test for homogeneity of variances at the 0.05 level of probability (Douillet, 1991).

In Experiment V, oyster larvae were fed IA every second day according to the methods employed with live algae in Experiments I to III. Three groups of larval cultures were fed IA. One group was maintained bacteria-free, while the two others were inoculated at the beginning of the experiment with strains H7 or CA2. Control treatments included starved larvae or larvae fed every two days on live axenic *Isochrysis galbana*. Eight replicates were tested per treatment. Larval survival and growth were determined as described below.

Data collection and analysis

At the end of each experiment, the larvae were carefully transferred to scintillation vials containing buffered formaldehyde (2% final concentration, pH = 8). The larval tissues were stained with rose of Bengal, so that the larvae that were alive could be distinguished from empty shells. The whole larval population in each flask was counted with a dissecting microscope, and the shell lengths of 100 randomly selected larvae were measured, either with an optical micrometer fitted to a compound microscope, or with an image analysis system (Zeiss Videoplan 2). Survival and growth data were transformed to satisfy assumptions of ANOVA. Survival data were transformed as:

$$\text{arcsin (square root (percent survival } 100^{-1}))$$

Growth data were transformed as:

$$\text{arcsin (square root ((ln } L_t - \text{ln } L_0)t^{-1}))$$

where L_t is the final mean shell length (μm); L_0 is the initial mean shell length (μm); and t is the culture period (10 days).

These transformations were successful in reducing the heterocedasticity of the survival data but not of the growth data (Cochran's test for heterogeneity of variances, at the 0.05 level of probability). Treatment effects on larval survival were tested with one-way ANOVA. Where significant differences were indicated, Tukey's honestly significant difference test (T-HSD) was applied to determine the statistical significance of differences among individual treatments at the 0.05 level of probability. Treatment effects on larval growth were analyzed with the Kruskal-Wallis test (KW). Differences among individual treatments were determined by means of the Games and Howell test (G&H) of equality of means with heterogeneous variances (Sokal and Rohlf, 1981), at the 0.05 level of probability. All tests were performed with the computer program Statistix (NH Analytical Software), except the Games and Howell test which was carried out with the program Biom (Rohlf, 1982).

The size-frequency distributions of populations of algae-fed larvae that were bacteria-free were compared with those fed algae supplemented with CA2 bacteria in Experiments II and III. Skewness coefficients (g1; Sokal and Rohlf, 1981) of larval populations from each replicate flask were calculated and used to compare larval size frequency distributions. A normal size distribution would have a g1 coefficient equal to 0. A skewness coefficient higher than 0 indicates that the size distribution is positively skewed (higher proportion of small-sized individuals), while a coefficient smaller than 0 indicates negative skewness. After confirmation of homocedasticity of g1 values by Cochran's test at the 0.05 probability level, data were analyzed by two-way ANOVA with treatment (algae, algae + CA2) and experiment as factors. As dictated by the results of ANOVA, appropriate multiple comparisons of means were conducted at the 0.05 level of probability using the Student-Newman-Keuls procedure (SNK), controlling for experiment-wide error (Underwood, 1981).

Cryopreservation of bacteria

Bacteria have been described as adaptable chimaeras, the metabolic plasticity of which results from widespread transfer of genetic information though plasmids or prophages (Sonea, 1988). This strategy for adaptation to changing environments may result, during evolution, in the loss of beneficial characteristics of selected bacterial strains. In order to reduce the possibility of changes in bacterial characteristics between successive experiments, selected strains were cryopreserved at −70°C in 10% (V/V) glycerol in sterile 1/10 diluted marine broth 2216.

Identification of strain CA2

The identification of bacterial strain CA2 was based on *Bergey's Manual of Systematic Bacteriology* (Holt, 1984). The methodology used for different procedures followed the *Manual of Methods of General Bacteriology* (Gerhardt *et al.*, 1981). Exponentially growing cells cultured on marine agar 2216 were used for the following tests performed at the Hatfield Marine Science Center, Newport, Oregon. (a) Cells were Gram stained. (b) Motility was determined by observations of wet mounts with light microscopy. (c) Oxidase activity was determined by spreading CA2 cells with sterile cotton swabs over Pathotec cytochrome oxidase test strips (General Diagnostics), which contained a derivative of dimethyl-p-phenylenediamine and α-naphthol. (d) Cultures of CA2 cells were flooded with 3% hydrogen peroxide for catalase testing. (e) Oxidation and fermentation of glucose was assayed with the modified O-F medium of Leifson (1963). (f) Utilization of inorganic

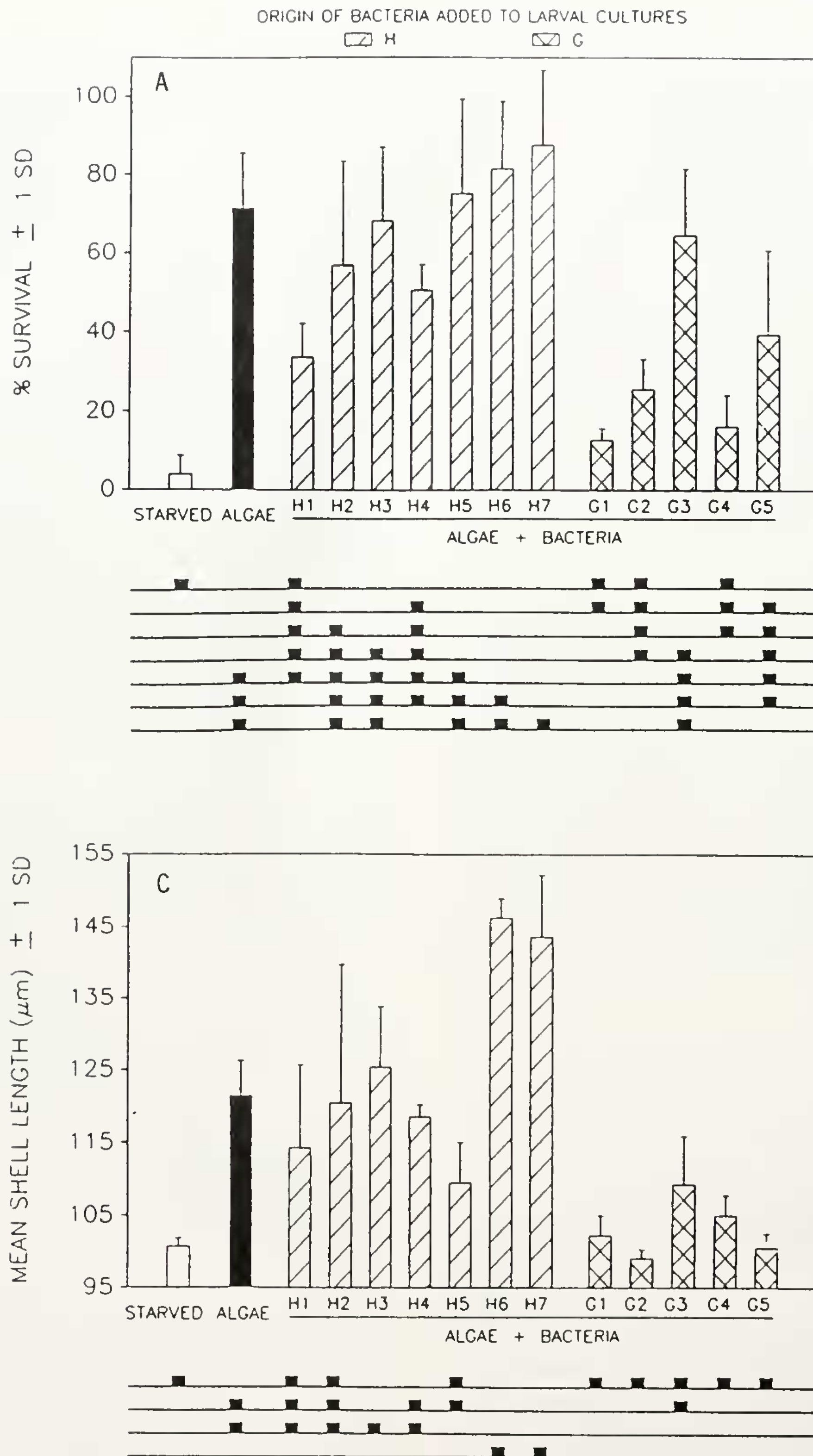

Figure 1. Effects of different bacterial strains on oyster larvae cultured on a diet of axenic *Isochrysis galbana* for 10 days. (A) Survival in Experiment I. (B) Survival in Experiment II. (C) Growth in Experiment I. (D) Growth in Experiment II. Bacteria were isolated from the Whiskey Creek Hatchery, Oregon (H), from the guts of adult oysters (G), from incubations of protein capsules in seawater (CA), or were naturally-occurring in 1 μm-filtered seawater (SW). Larval control treatments were starved or fed axenic *I. galbana*. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below the histograms of survival and growth, respectively. Squares that occur together on any one of the horizontal lines indicate mean values that are not different at the 0.05% level of significance.

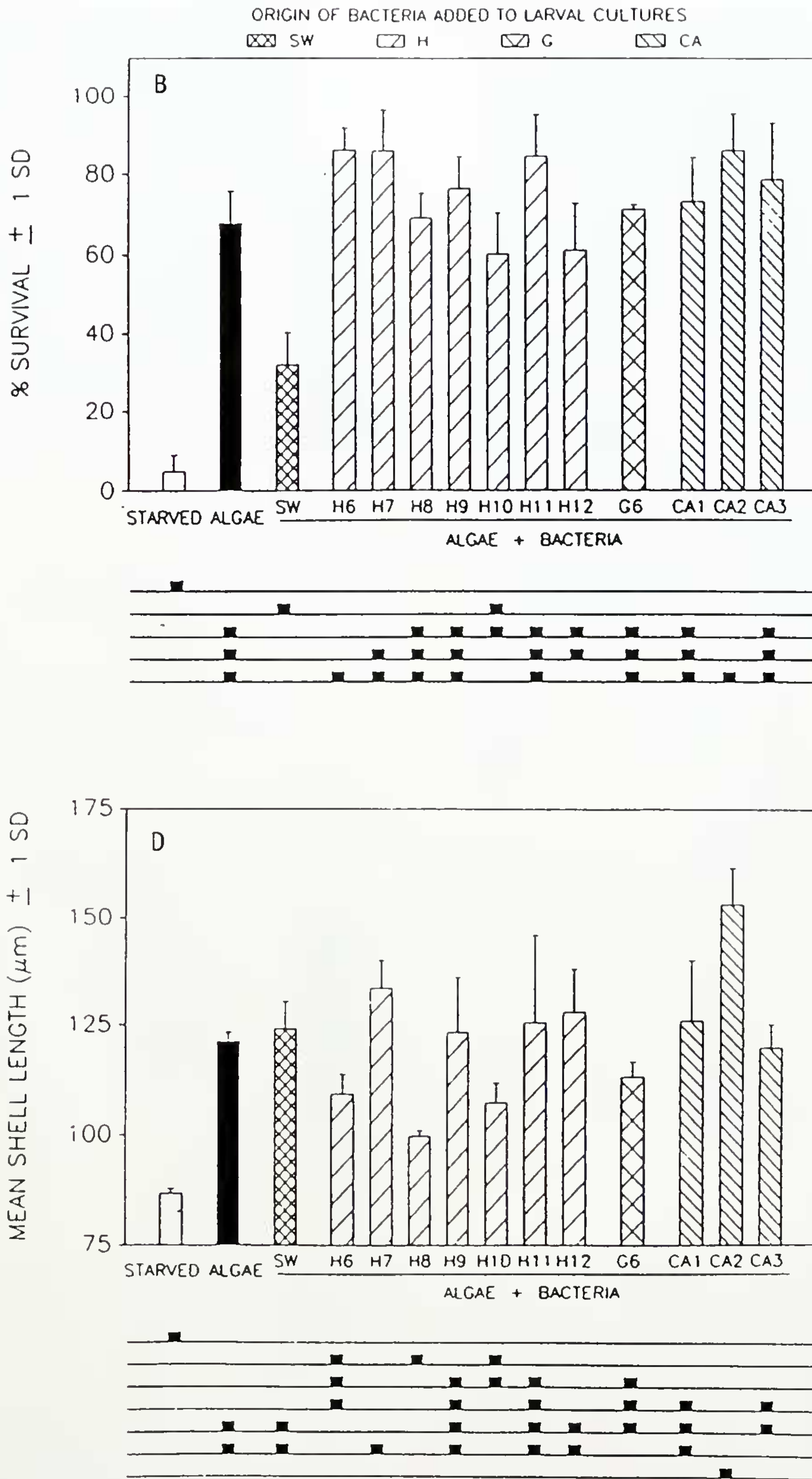

Figure 1. (*Continued*)

sources of nitrogen was evaluated by culturing CA2 cells on media prepared with NH_4Cl or $NaNO_3$ (0.5 g l^{-1}), glucose (0.1 g l^{-1}), Na_2HPO_4 (0.1 g l^{-1}), $FePO_4$ (0.004 g l^{-1}) and 1 ml l^{-1} of f/2 vitamin mix (Guillard and Ryther, 1962). The culture media used as controls were prepared by replacing $NaNO_3$ or NH_4Cl with peptone or tryptone (Difco) at 0.5 g l^{-1}. (g) Anaerobic growth was determined by transferring CA2 cells either into solid media in Petri dishes, or into 25 ml 1/10 diluted marine broth 2216 (3.74 g l^{-1}; salinity 30 ppt) contained in 50 ml Erlenmeyer flasks, placing these cultures in anaerobic GasPak pouches (BBL), and incubating the cells at 20°C for up to one month.

The following tests were carried out by Dr. Ronald Weiner (University of Maryland at College Park). Methodology followed the *Manual of Methods for General Bacteriology* (Gerhardt *et al.*, 1981). (a) Salt requirements were evaluated by culturing CA2 cells in tryptic soy agar (TSA) prepared at different salt concentrations; NaCl was added at 1% increments up to 10% of the control level. (b) As evidence of anaerobic growth and motility, CA2 cells on a straight needle were used to inoculate a tube containing semisolid tryptic soy broth enriched with 0.8% agar and 1% NaCl, and the pattern of growth observed. (c) Flagellar staining was carried out by the Leifson method (Gerhardt *et al.*, 1981). (d) Synthesis of exopolysaccharides was evaluated by the phenol-sulfuric acid reaction (Gerhardt *et al.*, 1981). (e) The mole percent guanine plus cytosine (mol% G + C) in extracted deoxyribonucleic acid (DNA) was determined by the thermal melting (denaturation) methods of Marmur and Doty (1962) with a Gilford UV programmable spectrophotometer. (f) Antibodies of 20 different bacteria strains belonging to the Alteromonas/Shewanella group were tested for reaction with exopolysaccharides of CA2 cells. (g) Fatty acid analyses of strain CA2 were carried out for comparison with profiles of other marine bacteria by Dr. Fred Singleton (Center for Marine Biotechnology, University of Maryland) and by Dr. Warren L. Landry (Food and Drug Administration, Dallas, Texas).

Results

Larvae fed on live algae and bacteria

Single additions of marine bacterial isolates to oyster larvae cultures significantly affected larval survival (ANOVA, $P < 0.01$) and growth (KW, $P < 0.01$) after 10 days of culture in all experiments (Figs. 1, 2). The microbes tested can be divided into categories depending on their effects upon oyster larvae: adverse, neutral, or beneficial. Bacteria belonging to the last category were tested further, and their effects upon oyster larvae were designated as either variable or consistently beneficial.

Adverse strains. Strains G1, G2 and G4 adversely affected larval survival (T-HSD, $P < 0.05$), whereas strains G1, G2, G4, G5, H8, and H10 adversely affected larval growth (G&H, $P < 0.05$). Bacteria present in 5 ml aliquots of 1 μm-filtered seawater depressed larval survival (T-HSD, $P < 0.05$) in Experiment II and larval growth (G&H, $P < 0.05$) in Experiment III.

Neutral strains. A large proportion of the strains (H1, H2, H3, H4, H5, H9, H11, H12, G3, CA1, and CA3) added to cultures of oyster larvae had no significant effect on larval survival (T-HSD, $P > 0.05$) or growth (G&H, $P > 0.05$) compared with cultures fed algae alone.

Variable strains. Addition of strains H6 and H7 to larval cultures caused inconsistent improvements of larval growth. For example, larval growth was enhanced (G&H, $P < 0.05$) in cultures inoculated with strains H6 and H7 in Experiment I, but the enhancement with strain H7 was statistically insignificant in Experiments II and III (G&H, $P > 0.05$). Moreover, larval growth was depressed (G&H, $P < 0.05$) when strain H6 was added to larval cultures in Experiment II.

Beneficial strains. In both Experiments II and III, larvae grown in cultures inoculated with strain CA2 had a significantly greater shell length than control larvae fed only axenic algae (G&H, $P < 0.05$). Larval survival was enhanced in cultures inoculated with strain H7 and CA2, but this enhancement was statistically significant only in Experiment III (T-HSD, $P < 0.05$).

Size frequency distributions of populations of larvae fed axenic algae were skewed compared to those from cultures fed algae supplemented with CA2 bacteria (Fig. 3; Table 1). Analysis of variance indicates a significant interaction between treatment and experimental factors (Table 2). In both Experiments II and III, skewness coefficients for populations of larvae fed axenic algae alone were significantly larger (SNK, $P < 0.05$) that those for populations of larvae fed algae and inoculated with CA2 bacteria. The difference between the skewness coefficients of treatments in Experiment II is larger than that in Experiment III, explaining the significant interaction determined by the two-way ANOVA test.

Effects of CA2 bacteria on the growth of algae in larval cultures

Cells of *Isochrysis galbana*, with or without inoculations of CA2 bacteria, did not grow under the conditions used to culture larvae (Fig. 4). The occurrence of CA2 cells in the culture medium had no effect on algal growth under favorable light intensity (1000–1500 lux) and temperature (20°C).

Larvae fed on dead algae and bacteria

Significant differences among treatments in Experiments IV and V were determined for larval survival (ANOVA, $P < 0.01$) and growth (KW, $P < 0.01$). The survival of larvae cultured on axenic FA or IA alone was significantly lower (T-HSD, $P < 0.05$) than that of larvae

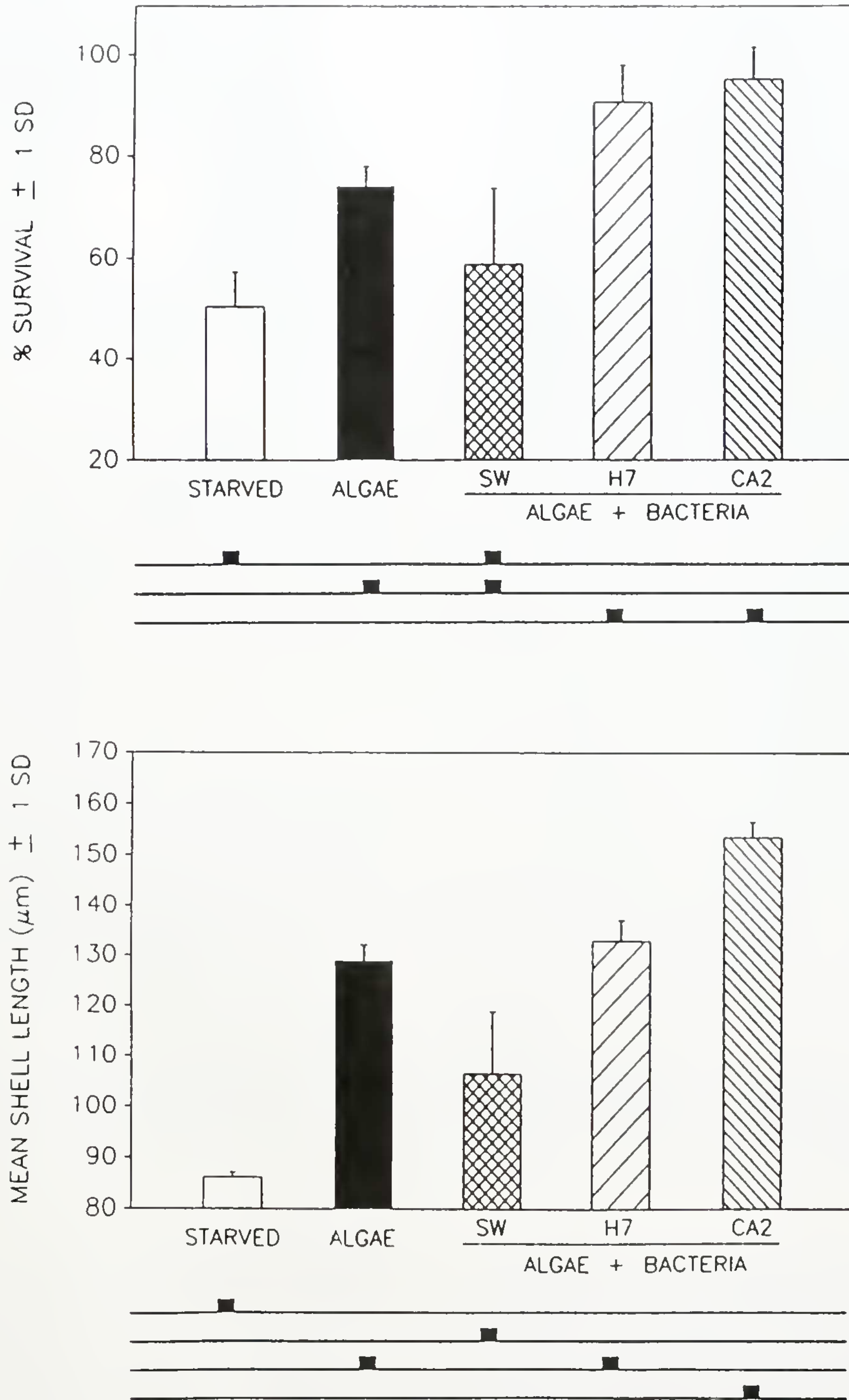

Figure 2. Survival and growth of oyster larvae after 10 days of culture on axenic *Isochrysis galbana* supplemented with different bacterial strains (Experiment III). Bacteria were isolated from the Whiskey Creek Hatchery, Oregon (H) or from incubations of protein capsules in seawater (CA). Naturally-occurring bacteria present in 1 μm-filtered seawater (SW) were added in a control treatment. Other control treatments included larvae fed axenic *I. galbana* or starved. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below the histograms of survival and growth, respectively. Squares that occur together on any one of the horizontal lines indicate mean values that are not different at the 0.05% level of significance.

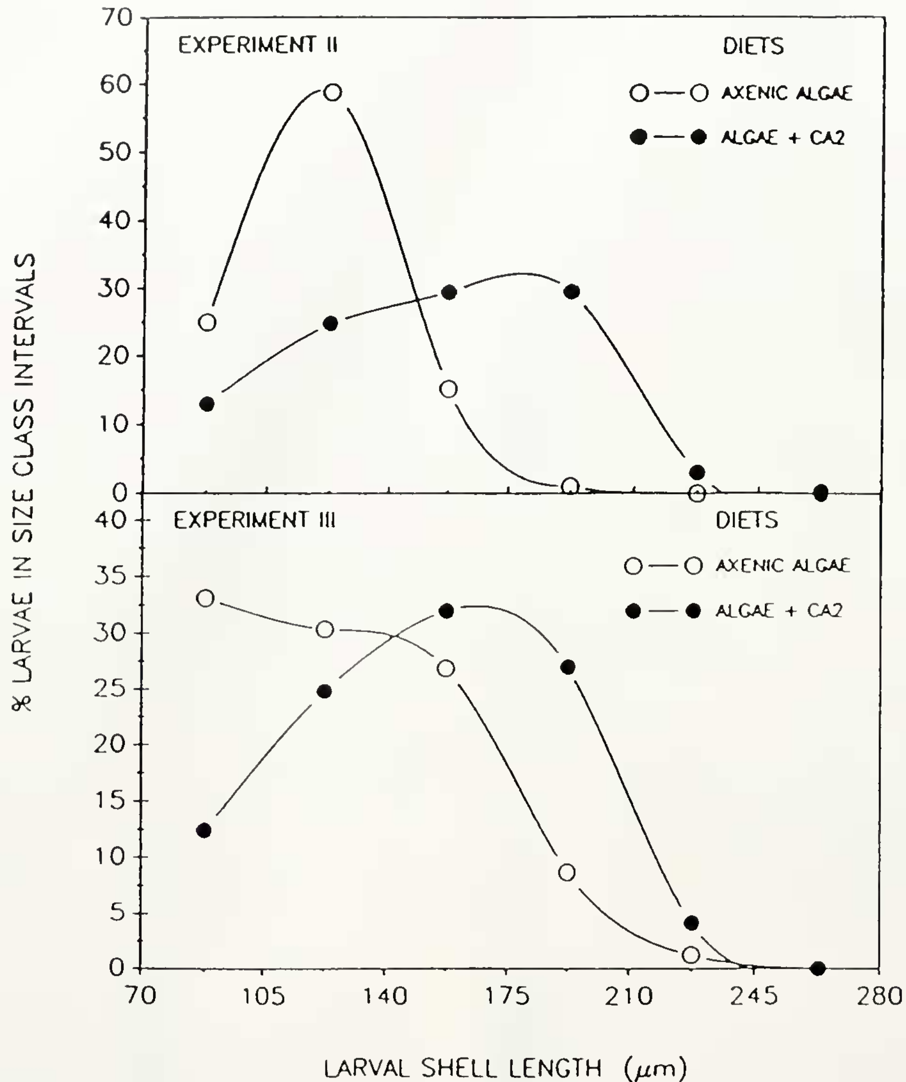

Figure 3. Size frequency distributions of larvae cultured for 10 days on a diet of *Isochrysis galbana* with or without addition of CA2 cells. Points represent percent larvae for each shell length interval of 30 μm. Lines used for illustrative purposes only. Data from Experiments II (n = 400) and III (n = 800) for each treatment.

cultured on live axenic algae alone (Figs. 5, 6). However, the survival of larvae fed FA or IA was higher (T-HSD, $P < 0.05$) than that of starved larvae. In contrast, no significant differences in larval survival were detected between cultures fed live algae and cultures fed FA or IA inoculated with strains H6 and H7, respectively (T-HSD, $P > 0.05$). Survival of larvae fed every two days on bacteria H6 alone was not significantly different (T-HSD, $P > 0.05$) from that of larvae fed live algae, and was significantly higher (T-HSD, $P < 0.05$) than that of starved larvae (Fig. 5). Larvae from cultures inoculated every two days with 5 ml of 1 μm-filtered seawater (SW) also showed higher survival (T-HSD, $P < 0.05$) than that of starved larvae.

Larvae fed on FA or IA were significantly smaller than larvae fed on live axenic algae (G&H, $P < 0.05$), and were not different from the size of starved larvae (G&H, $P > 0.05$) at the end of the experiment (Figs. 5, 6). Additions of single bacterial strains to cultures of larvae fed FA or IA did not improve larval growth compared to larvae fed FA or IA alone (G&H, $P > 0.05$). In contrast, growth of larvae fed FA inoculated with 5 ml of 1 μm-filtered seawater was significantly enhanced (G&H, $P < 0.05$) compared to that of larvae fed FA alone or starved larvae (Fig. 5). Similarly, additions every two days of 5 ml of 1 μm-filtered seawater or strain H6 alone to larval cultures significantly enhanced the growth of larvae (G&H, $P < 0.05$) compared to that of starved larvae.

The poor growth of larvae fed FA may have been due to the rupture of the freeze-killed algal cells. ^{60}Co-irradiation did not affect the integrity of the algal cells but re-

Table I

Skewness coefficients (g1) from size frequency distributions of populations of larvae cultured in Experiments II and III

Experiment	Diet	Average skewness of populations ± 1 S.D.
II	ISO	0.7906 ± 0.2134 (n = 4)
II	ISO + CA2	−0.0605 ± 0.2235 (n = 4)
III	ISO	0.3801 ± 0.1720 (n = 8)
III	ISO + CA2	−0.0466 ± 0.2910 (n = 8)

Larvae were cultured with either axenic *Isochrysis galbana* (ISO) alone or *I. galbana* plus CA2 bacteria.

duced their volume from 44.4 ± 1.92 μm^3 to 26.3 ± 0.59 μm^3 ($\bar{x}$ ± 1 SD; n = 7). A high proportion of irradiated cells remained intact while in suspension in seawater, as demonstrated by the small decrease in cell concentration in control flasks, from 59,043 ± 1,119 cells ml^{-1}, to 58,539 ± 1,505 cells ml^{-1} ($\bar{x}$ ± 1 SD; n = 4) in 105 min. IA cells were ingested by oyster larvae at rates significantly (2 sample t-test, $P < 0.01$) greater than that for live cells.

Identification of strain CA2

Strain CA2 was presumptively identified as *Alteromonas* sp. on the basis of the following characteristics: Gram negative rod; aerobic; oxidase positive; requires 250 n*M* salt; motile with polar flagella; exopolysaccharide synthesis; and guanine plus cytosine 43 mol% (T_m).

The exopolysaccharides of CA2 bacteria did not react with antibodies to 20 species of *Alteromonas*. Furthermore, both analyses of fatty acids revealed a very unusual fatty acid profile with a high proportion of C-14, C-15 fatty acids (Table 3); this is not characteristic of the genus *Alteromonas*. However, the fatty acid profile was not similar to any of the species profiles listed in Dr. Landry's marine library. Therefore, strain CA2 may be an *Alteromonas* species not typical of the genus.

Further characteristics of strain CA2 include yellow pigment production, oxidation and fermentation of glucose, but no gas production, and inability to utilize inorganic sources of nitrogen, such as NH_4Cl or $NaNO_3$ for growth. Catalase was weakly positive.

Discussion

Axenic larval *Crassostrea gigas* were used to determine the effects of additions of single bacterial strains on the survival and growth of larvae cultured with algae. Bacteria can be categorized as adverse, neutral or beneficial, depending on their effects upon oyster larvae. Furthermore, bacteria found beneficial in one experiment were retested in subsequent experiments and could be further categorized as either variable or consistently beneficial strains. Additions of strain CA2 to larval cultures consistently enhanced larval survival (21–22%) and growth (16–21%) compared with that of larvae fed on algae alone.

The specificity of bacterial strains as food for grazers has frequently been reported (Frosch, 1897 in Luck *et al.*, 1931; Stuart *et al.*, 1931; Curds and Vandyke, 1966). Furthermore, Curds and VanDyke (1966) found that one bacterial strain was either slightly toxic, unfavorable, or favorable depending on the ciliate species tested. In contrast, a single bacterial strain (PM-4) was found to promote the growth of both shrimp (*Penaeus monodon*) and crab (*Portunus tridentatus*) larvae (Maeda, 1988; Maeda and Nogami, 1989). Consequently, no generalization about the beneficial effects of specific bacterial strains can be made; *i.e.*, each strain must be tested again with each new target species.

Bacteria may be used directly as a food item by oyster larvae (Douillet, 1991). Starved axenic oyster larvae showed poor survival and did not grow after 10 days of culture. In contrast, larvae in cultures inoculated with single bacterial strains or mixtures of naturally-occurring marine bacteria had higher survival rates than starved larvae, but lower growth rates than larvae fed on algal diets. Consequently, the bacterial strains tested did not provide all the nutritional requirements for larvae, but appeared, at least, to partially satisfy larval metabolic requirements, as demonstrated by the beneficial effects of bacteria on larval survival and growth. Straight-hinged oyster larvae, fed for 10 min on ^{14}C-labeled CA2 cells at 1.5×10^7 cells ml^{-1} and purged of undigested ^{14}C-material, retained enough bacterial carbon to meet over 140% of their active carbon metabolic requirements during a 10 min period (Douillet, 1991). Beese (in Prieur *et al.*, 1990) determined that xenic, starved larval *Crassostrea gigas* grew 60% in size after seven days of culture, whereas starved axenic larvae did not grow. The ability of starved xenic bivalve larvae to grow has been determined to be greater for larvae

Table II

Two-way analysis of variance of skewness coefficients (g1) for size frequency distributions of populations of larvae cultured in Experiments II and III

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment (A)	1	0.31468	0.31468	5.79	0.0259
CA2 addition (B)	1	3.2656	3.2656	60.12	0.0000
Interactions (A*B)	1	0.36039	0.36039	6.63	0.0180
Replicates (C)					
Residual (A*B*C)	20	1.0864	0.05432		
Total	23	5.0271			

Larvae were cultured with *Isochrysis galbana* alone or *I. galbana* plus CA2 bacteria.

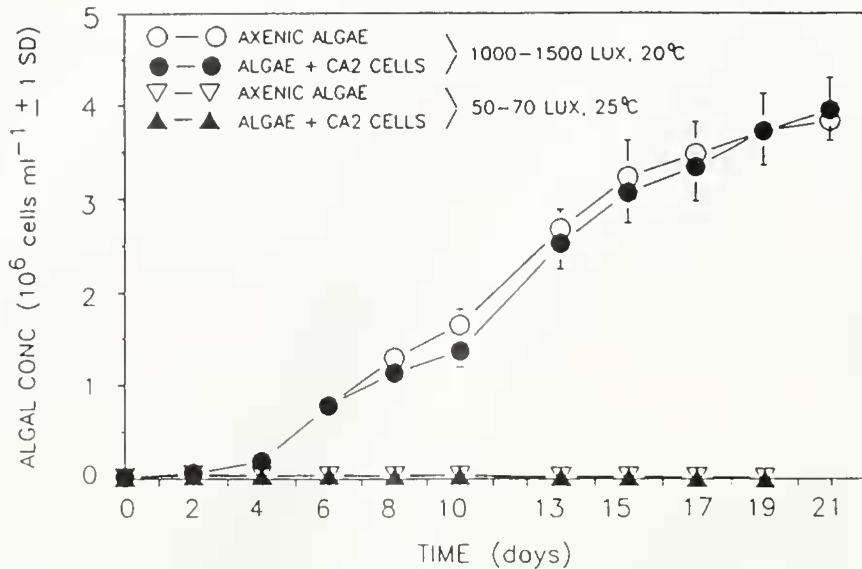

Figure 4. Effects of CA2 bacteria on growth of *Isochrysis galbana* under conditions used to raise larvae (50–70 lux; 25°C) or under conditions found optimal for algal growth (1000–1500 lux; 20°C).

of the mussel *Mytilus edulis* than for larval *C. gigas* (His *et al.*, 1989). But bacteria lack long-chain polyunsaturated fatty acids (PUFA) (Kates, 1964; Perry *et al.*, 1979) and sterols (Lehninger, 1975), both of which may be essential for the growth of marine bivalves (Trider and Castell, 1980; Langdon and Waldock, 1981). This lack of essential nutrients could explain why larvae grew more poorly on a diet of bacteria alone than on a diet of algae alone.

Size-frequency distributions of bacteria-free oyster larvae cultured for 10 days on axenic live algae were always positively skewed due to a high proportion of larvae that exhibited poor growth. Algae were always present in cultures at satisfactory concentrations for larval growth (Breese and Malouf, 1975); therefore, the poor growth of some larvae in populations fed axenic algae could not be due to insufficient algal food. In contrast, additions of CA2 bacteria to cultures of algae-fed larvae consistently normalized larval size-frequency distributions. Larval survival was equal (Experiment II; Fig. 1B, D) or higher (Experiment III; Fig. 2) in cultures inoculated with strain CA2 than in cultures fed algae alone; therefore, changes in size-frequency distributions were not due to the selective death of slow growing larvae in bacterized cultures. Instead, additions of strain CA2 to larvae fed on algae apparently shifted larval size-frequency distributions by promoting the growth of larvae that would grow poorly on an algal diet alone. This result suggests that some oyster larvae in cultured populations require supplements of bacteria in order to grow, and that an algal diet of *Isochrysis galbana* alone is not sufficient to meet their nutritional requirements.

The inability of a single algal food species to support larval growth rates comparable to those obtained on mixtures of algal species suggests that diets of single algal species can be nutritionally inadequate for maximum larval growth (Davis and Guillard, 1958; Walne, 1970). Microbes could provide dietary micronutrients, such as vitamins (Kutsky, 1981) or other growth factors, that could be deficient in algal diets. Vitamin deficiencies in the media used to culture axenic *Artemia* have arrested growth and caused the early mortality of this crustacean (Provasoli and D'Agostino, 1962). Vitamin supplements increased the growth rate of larval *Crassostrea virginica*, when given alone or in combination with *Chlorella* (Davis and Chanley, 1956). The high nutritional value of bacteria is indicated by the success of bacterial supplements in improving the quality of algae (Provasoli *et al.*, 1959), or of dried diets of different chemical composition (Douillet, 1987).

Bacterial enhancement of larval cultures may also have been due to other mechanisms apart from bacterivory. Bacteria could have acted as a symbiont for larvae, contributing to the larva's protein nutrition through nitrogen fixation (Benemann, 1973; Carpenter and Culliney, 1975; Guerinot and Patriquin, 1981), or by aiding in the digestion and assimilation of ingested algae. The bacterial flora of bivalve larvae consists of a high proportion of strains that produce extracellular enzymes, such as proteases and lipases (Prieur, 1982).

Oyster larvae were grown for 10 days with no change in the culture medium; thus metabolites excreted by bivalves (Cockcroft, 1990) and algae (Hellebust, 1974) would accumulate in the larval cultures. Strain CA2 may

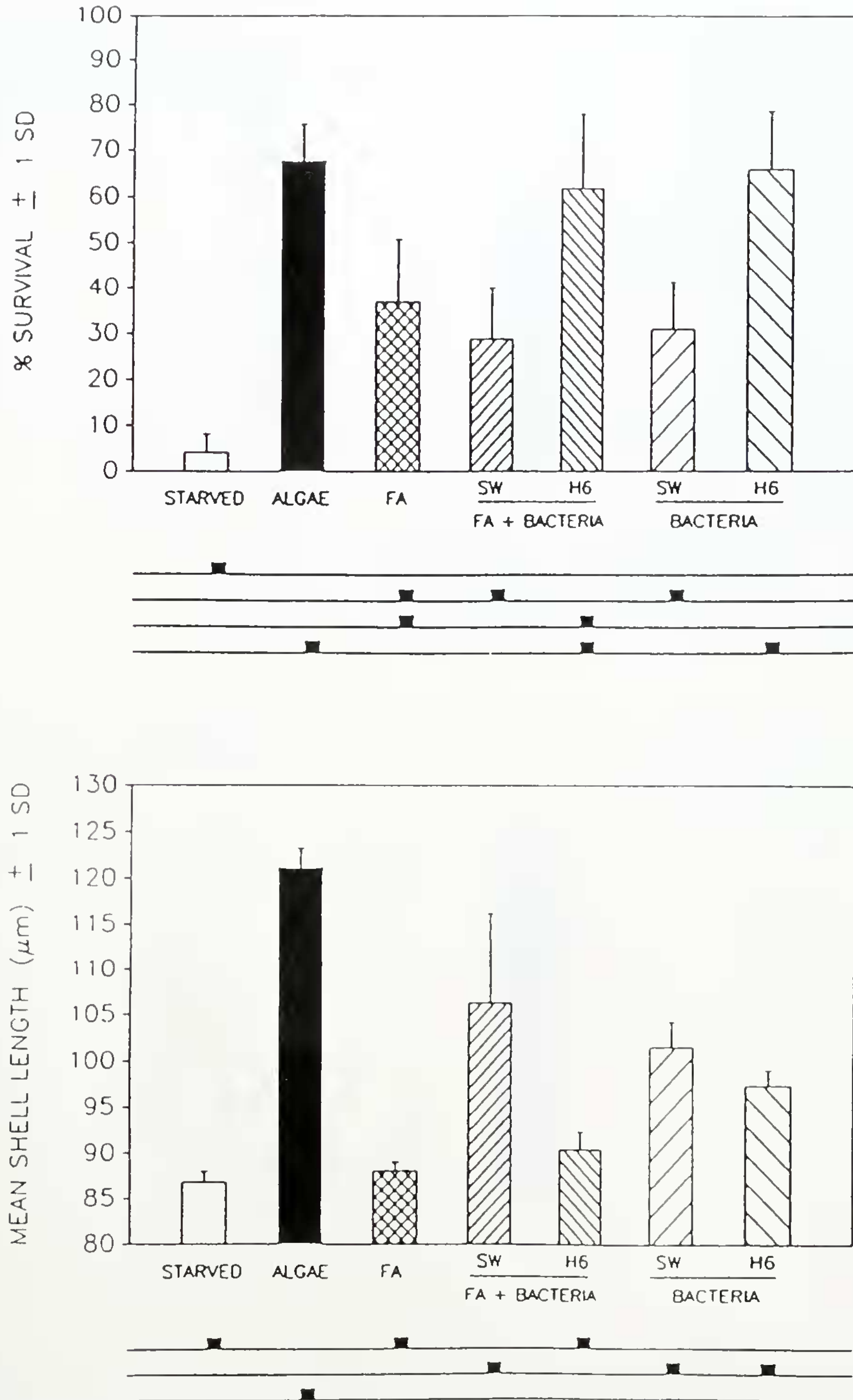

Figure 5. Survival and growth of oyster larvae after 10 days of culture when fed on a diet of either bacteria alone (strain H6, naturally-occurring bacteria present in 1 μm-filtered seawater (SW)) or freeze-killed *Isochrysis galbana* (FA) with or without supplements of bacteria (H6 or SW) (Experiment IV). Control treatments were starved or fed axenic *I. galbana*. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below survival and growth histograms, respectively. Squares that occur together on any one of the horizontal lines indicate mean values that are not different at the 0.05% level of significance.

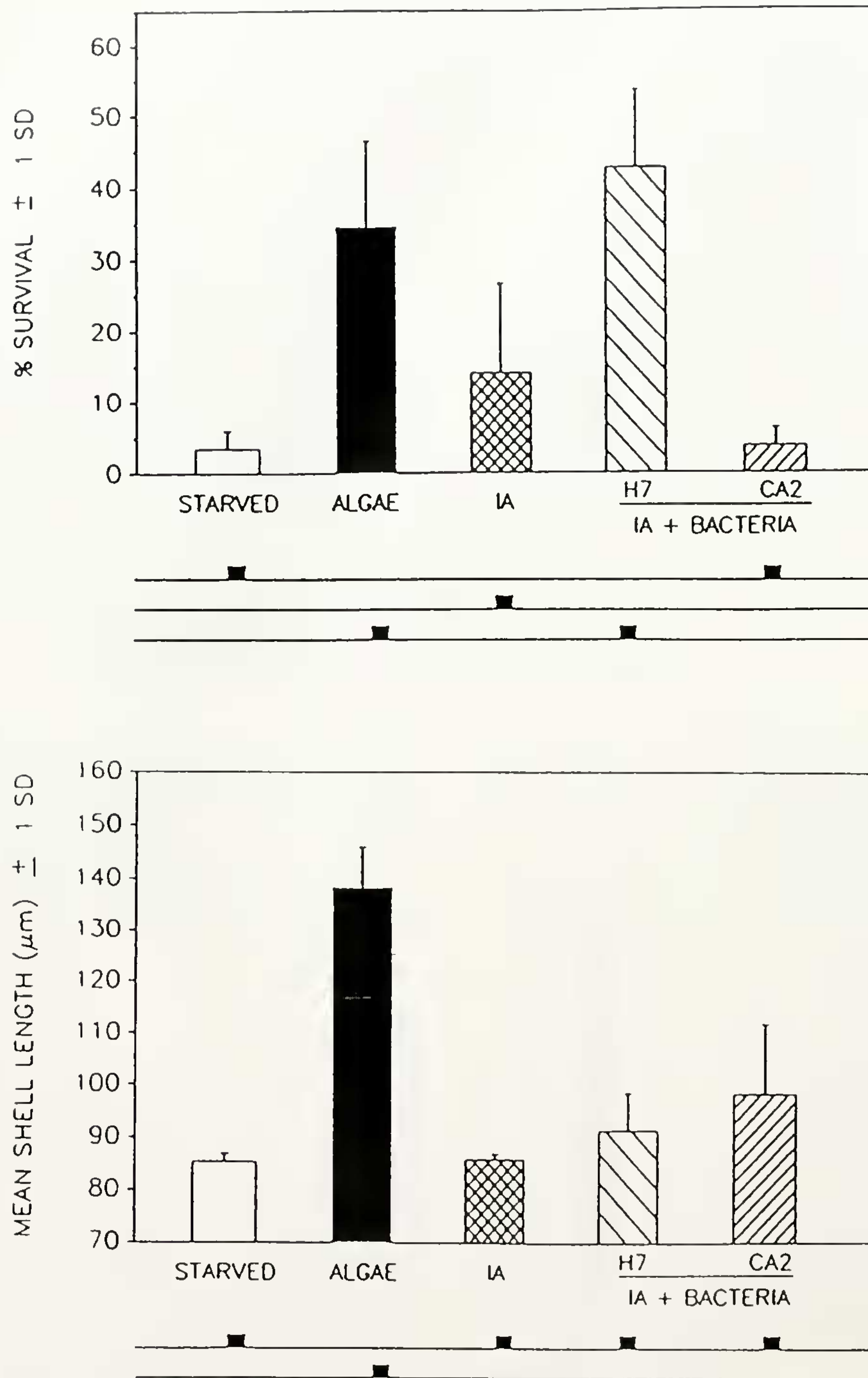

Figure 6. Survival and growth of oyster larvae after 10 days of culture on ^{60}Co-irradiated *Isochrysis galbana* (IA) with or without supplements of H7 and CA2 bacteria (Experiment V). Control treatments were starved or fed axenic *I. galbana*. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below survival and growth histograms, respectively. Squares that occur together on any one of the horizontal lines indicate mean values that are not different at the 0.05% level of significance.

have enhanced larval cultures by removing toxic metabolites. This may have stimulated larval growth and may have normalized the size-frequency distribution by promoting the growth of larvae that were more sensitive than others to the adverse growth effects of metabolites. However, the growth of xenic larvae was also enhanced by the addition of CA2 bacteria in cultures where the water was replaced every second day (Douillet, 1991); therefore,

Table III

Fatty acid composition of CA2 bacteria

Fatty acid	% composition
Unknown 11.541	1.73
14:0 ISO	4.17
14:0	0.76
15.1 ISO G	4.99
15:0 ISO	18.99
15:0 ANTEISO	8.07
15:1 B	6.62
15:0	4.66
16:1 ISO H	5.71
16:0 ISO	2.02
16:1 CIS 9	4.08
16:0	0.99
15:0 ISO 3OH	12.44
15:0 3OH	1.89
17:1 C	2.24
16:0 ISO 3OH	15.98
16:0 3OH	1.00
17:0 ISO 3OH	1.78
17:0 2OH	1.21

bacterial removal of toxic metabolites from culture waters is less likely the mechanism of enhancement of larval growth.

Strain CA2 did not indirectly affect larvae by increasing algal growth and food availability in larval cultures, because no enhanced algal growth occurred in the presence of CA2 bacteria.

Larvae did not grow when fed on freeze-killed or ^{60}Co-irradiated *Isochrysis galbana,* and additions of bacteria did not significantly improve the growth of larvae fed on either of the two killed algal diets. ^{60}Co-irradiated algal cells were grazed by larvae at rates that were significantly higher than those for live algal cells (Douillet, 1991). This suggests that the poor growth of larvae fed on killed algal diets was not due to a lack of available particulate matter, but more likely to the destruction or loss of essential nutrients from killed algal cells. Supplements of bacterial strains or mixtures of naturally occurring bacteria did not overcome these possible nutritional deficiencies of the killed algal diets.

The ability of the larvae of some bivalve species to utilize dead algae as food under xenic conditions has been well documented. Larvae of the mussel *Mytilus galloprovincialis* grew at similar rates whether fed on live or frozen *Monochrysis lutherii* (Masson, 1977). Chanley and Normandin (1967) reported that larvae of the clam *Mercenaria mercenaria* grew and survived equally well when fed on either live or frozen cells of *Isochrysis galbana.* However, different species of bivalves appear to have different nutritional requirements, as indicated by the findings of Loosanoff (1954) on the ability of *M. mercenaria* larvae to utilize a greater variety of natural foods than the larvae of the American oyster *Crassostrea virginica.* Larvae of *M. mercenaria* grew when fed on a diet of lyophilized *I. galbana* (Hidu and Ukeles, 1962) or frozen *I. galbana* (Chanley and Normandin, 1967), whereas larval *C. virginica* did not grow when fed either of these nonliving diets. The failure of larval *Crassostrea gigas* to grow when fed on dead algae impeded the evaluation of the direct nutritional contribution by bacteria under conditions where potential bacteria-algal interactions were eliminated by the use of killed, rather than living, algal cells.

In summary, bacteria added as single strains or as natural communities were found to be major sources of variation in cultures of *Crassostrea gigas* larvae. Selection of a consistently beneficial bacterium (strain CA2) for bivalve larval culture offers a valuable tool for research on the role of bacteria in the nutrition and culture of marine invertebrates. In addition, the use of beneficial microbes in aquaculture may contribute to the reduction of undesirable variation in culture success.

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